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Shared ITS DNA substitutions in isolates of opposite mating type reveal a recombining history for three presumed asexual species in the filamentous ascomycete genus *Alternaria*

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About 15 000 species of ascomycete fungi lack a known sexual state. For fungi with asexual states in the anamorph genera *Embellisia*, *Ulocladium*, and *Alternaria*, six species have known sexual states but more than 50 species do not. In sexual filamentous ascomycetes, opposite mating type information at the *MAT1* locus regulates mating and the opposite mating type genes each have a clonal, non-recombining phylogenetic history. We used PCR to amplify and sequence fragments of the opposite mating type genes from three supposedly asexual species, *A. brassicae*, *A. brassicicola* and *A. tenuissima*. Each haploid fungal isolate had just one mating type, but both mating types were present in all the three species. We sequenced the ribosomal ITS regions for isolates of opposite mating type, for the three asexual species and four known related sexual species. In a phylogenetic analysis including other ITS sequences from GenBank®, the three asexual species were not closely related to any of the known sexual species. Isolates of opposite mating type but the same species had identical ITS sequences. During any period of asexual evolutionary history, lineages of each mating type would have had a separate evolutionary history and any ITS substitutions shared between isolates of opposite mating type would have had to accumulate by convergence. Allowing for varying substitution rates and assuming a Poisson distribution of substitutions, the probability that isolates of opposite mating type shared an ITS substitution through convergence was low. This suggests that isolates of opposite mating type of *A. brassicae*, *A. brassicicola* and *A. tenuissima* were exchanging substitutions through sexual or parasexual reproduction while the ITS was evolving. If sexuality was lost, it was lost after the period of evolutionary history represented by the shared substitutions.

INTRODUCTION

An estimated half of the 30 000 or so species of fungi in the *Ascomycota* (ascomycetes) have only been observed in their asexual states (Hawksworth *et al.* 1995). Sexual states have only been linked to six (Simmons 1986, 1990) of the 50+ species of *Alternaria* and allies (Hawksworth *et al.* 1995), raising the possibility that the evolutionary history of this group involved the radiation and diversification of asexual taxa. Following taxonomic convention, the sexually-reproducing fungi in the form genera *Alternaria* and *Embellisia* also have formal botanical names, in the genera *Lewia* and *Allewia*, respectively and they are classified in the *Pleosporaceae* (Simmons 1986, 1990). Species in *Alternaria* with no known sexual state are common and economically significant plant pathogens. Because they are frequently cultured and because other aspects of their biology and physiology are well-studied (Chelkowski & Visconti 1992), the apparent lack of sexual reproduction in *Alternaria* species is not explained simply by lack of interest in finding it.

However, even though sexual reproduction has never been observed in most *Alternaria* species, asexuality may not have had a long evolutionary history, and it may not even be obligate. Genetic approaches have detected a history of recombination in several ascomycete species where sexual reproduction has never been seen (Burt *et al.* 1996, Graeser *et al.* 1996, Geiser, Pitt & Taylor 1998, Taylor, Jacobson & Fisher 1999). Burt *et al.* (1996), for example, found phylogenetic conflict among different genetic loci, strong evidence for historical recombination in the presumed asexual fungus *Coccidioides immitis*. There could be several explanations for the lack of observed sexual reproduction in species with a history of recombination. Obligate asexuality may have evolved frequently but been short-lived, so that the asexual populations retained the evidence of their sexual origins. Or possibly, recombination in fungal populations involved

parasexuality and not the formation of the usual organs of sexual reproduction. In the laboratory, many ascomycetes can undergo a parasexual cycle involving somatic fusion, diploidization and haploidization (Caten 1981). Parasexuality can reshuffle genetic material in fungi, in the absence of compatible mating types and without meiosis (Caten 1981). In nature, however, the vegetative incompatibility systems that prevent unlike fungal individuals from fusing might limit the importance of parasexuality (Caten 1981, Clutterbuck 1996). A further possibility is that conventional sexual reproduction occurs regularly in nature, but is difficult to detect because ascomycete sexual fruiting bodies are small and ephemeral. Laboratory testing often fails to detect sexual reproduction, possibly because the specific conditions needed to induce fruiting are hard to replicate.

When conventional sexual reproduction does occur in outcrossing ascomycetes, it begins with a cellular fusion between two compatible, haploid individuals. Fusion occurs only between individuals with unlike forms of the regulatory mating type genes. In a handful of filamentous ascomycetes, mating type genes have been cloned and characterized, as reviewed by Turgeon (1998) and by Shiu & Glass (2000). The genes found at the single *MAT1* locus encode proteins with a distinct DNA binding motif believed to function as regulatory elements with a broad influence (Turgeon & Berbee 1998). The DNA and amino acid sequences of the opposite mating type genes at the *MAT1* locus show no obvious similarities except for small islands of homology, although they are surrounded by common flanking regions (Turgeon *et al.* 1993). For fungi in the *Pleosporaceae*, the opposite genes can be designated '*MAT1-1-1*' and '*MAT1-2-1*' (Turgeon & Yoder 2000). *MAT1-1-1* and *MAT1-2-1* each have a clonal phylogenetic history that can be traced through the filamentous ascomycetes, and recombination between opposite mating type genes played no obvious role in their evolutionary history. Their evolutionary origin has been compared to the divergent evolution of sex chromosomes in higher eukaryotes (Metzenberg & Randall 1995, Turgeon 1998).

In the absence of sexual reproduction, release from selection might influence mating type gene evolution, leading to loss of genes or gene function. Surprisingly, structurally conserved mating type genes have been found in the few asexual species where they have been sought. Asexual species with conserved mating type genes include *Bipolaris sacchari* (Turgeon *et al.* 1993, Sharon *et al.* 1996), *A. gaisen* and *Fusarium oxysporum* (Arie *et al.* 1997, 2000), and the human pathogen *Candida albicans* (Hull & Johnson 1999). Furthermore, the *MAT1-2-1* gene from *B. sacchari* functioned to initiate mating when expressed in the closely related sexual species, *Cochliobolus heterostrophus* (Sharon *et al.* 1996). In *A. gaisen* (i.e. *A. alternata* 'Japanese pear pathotype') the two opposite mating type genes *MAT1-1-1* and *MAT1-2-1* at the *MAT1* locus have been cloned and characterized (Arie *et al.* 1997, Turgeon & Yoder 2000). Both the *MAT1-1-1* and *MAT1-2-1* genes from *A. gaisen* were expressed, as analyzed by reverse transcriptase PCR. Transformed into a *C. heterostrophus* isolate of the opposite mating type, expression of either gene from *A. gaisen* was able to stimulate initiation of pseudothecium and ascospore formation (Arie *et al.* 2000). During any period of asexual evolutionary history, lineages of each mating type would have had a separate evolutionary history and any shared DNA substitutions between isolates of opposite mating type would have had to accumulate by convergence (Fig. 1, hypothesis 2). Alternatively, during periods of sexual recombination, isolates of opposite mating type would exchange nucleotide substitutions (Fig. 1, hypothesis 1). Allowing for varying substitution rates and assuming a Poisson distribution of substitutions, the probability that isolates of opposite mating type accumulated their shared substitutions through convergence (as required by asexual evolution) could be estimated.

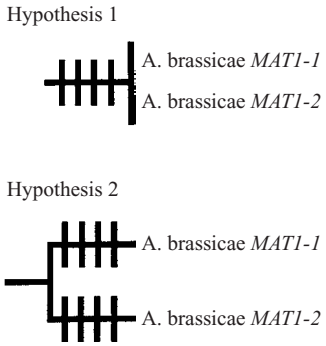

Fig. 1. How did strains with opposite mating types evolve the same ITS substitutions? In hypothesis 1, substitutions were exchanged across mating types, through a history of recombination. In hypothesis 2, the fungi were asexual and not recombining during their period of evolution. In hypothesis 2, the substitutions shared by members of the same species of opposite mating type would have had to evolve convergently. Hypothesis 2 could be rejected if the overall probability of substitution were low and recombination, rather than convergence, were the more probable explanation for the pattern of shared substitutions.

To evaluate substitution patterns, we planned to analyze ITS sequences, which were already available for many *Alternaria* species (Pryor & Gilbertson 2000). We planned to sequence the ITS regions of three 'asexual' species; *A. brassicae*, *A. brassicicola*, and isolates of the *A. tenuissima* group. We also selected four known sexual species for ITS sequencing, so that with sequences from the only two other known sexual species already in GenBank®, all known sexual species in *Lewia* and *Allewia* were represented in our analysis. Through a phylogenetic analysis, we could recognize whether any of our 'asexual' isolates were closely related to a known sexual species.

Our first goal was to look for both mating types among the supposed asexual isolates of *A. brassicae*, *A. brassicicola*, and of the *A. tenuissima* group. Our

second goal was to evaluate phylogenetically whether the mating type genes from the three *Alternaria* species were homologues to known mating type genes from other species. Our third goal was to explore whether substitutions in the ITS regions were shared by fungal isolates of opposite mating type in each species. By evaluating the extent to which ITS substitutions were shared between mating types, we could estimate whether recombination took place while ITS substitutions evolved.

MATERIALS AND METHODS

Fungal isolates

Alternaria brassicae and *A. brassicicola* were isolated by incubating leaf spots on cabbage leaves several days in moist chambers (Table 1). Conidiophores and conidia were found using a dissecting microscope at $\times 50$ magnification. Between 8 and 18 single conidia were isolated from each leaf spot and transferred to potato dextrose agar (PDA). Each of the isolates was designated by a collection number giving the initials of the author who isolated it, followed by the year of isolation ('01'), by a number specifying the particular leaf spot that served as the source of the series of isolates, and finally, a number specifying the particular conidial isolate (Table 1). The *A. brassicae* isolates gz01-3-1 through gz01-3-9 were from over-wintered cabbage from Acadia Park community gardens, University of British Columbia (UBC), Vancouver, BC (Table 1). Isolates gz01-6-1 through gz01-6-12 were from commercially sold cabbage of unknown origin (purchased March 2001 from The Real Canadian Superstore, in Vancouver, BC). The *A. brassicicola* isolate series gz01-2-1 through gz01-2-25 and gz01-8-4 through gz01-8-13 were from over-wintered cabbage leaves collected in February 2001 from near the Totem Park Climatological Station, UBC, Vancouver, BC (Table 1). Dried cultures and the original specimens on leaves were deposited in the UBC herbarium. The 28 *Alternaria tenuissima* group isolates examined were originally collected from 1991–1993, from cherry fruit without obvious injury of disease, from three orchards within 40 km of Wenatchee, WA (Table 1; Dugan & Roberts 1994). Isolates from species with known sexual states were obtained from the collection of Emory G. Simmons (Table 2). Cultures were maintained on V8 agar (Centraalbureau voor Schimmelcultures 1990) by refrigeration at 5 °C. Collections beginning with 'gz' used for sequence analysis were deposited in the University of Alberta Microfungus Collection and at the UBC Canadian Centre for the Culture of Microorganisms (Table 1). The other cultures are maintained by M. Berbee at the Department of Botany at UBC. Collections beginning with 'st' are maintained by R. Roberts at the USDA, ARS, Tree Fruit Research Laboratory in Wenatchee, WA. DNA was extracted (Lee & Taylor 1990) from isolates grown on PDA or V8 agar medium.

Table 1. List of mating type gene fragments detected in isolates of three putative asexual species of *Alternaria*.

Species	Isolate(s)	Collection no.	Mating type	GenBank® accession no.
A. brassicae	1	gz01-3-1	*MAT1-1-1*	AY042094
	2–9	gz01-3-2–9	*MAT1-1-1*	NA
A. brassicae	1–3	gz01-6-1 through gz01-6-4	*MAT1-2-1*	NA
	4	gz01-6-6	*MAT1-2-1*	AY042091
	5–8	gz01-6-8–12	*MAT1-2-1*	NA
A. brassicicola	1	gz01-2-1	*MAT1-1-1*	AY042093
	2–18	gz01-2-2–25	*MAT1-1-1*	NA
A. brassicicola	1	gz01-8-4	*MAT1-2-1*	NA
	2	gz01-8-6	*MAT1-2-1*	AY042092
	3–8	gz01-8-7–13	*MAT1-2-1*	NA
A. tenuissima	2	ST11-66	*MAT1-1-1*	NA
	3	ST8-42	*MAT1-1-1*	AY004675
	4	ST9-52	*MAT1-1-1*	NA
	5	ST11-61a′	*MAT1-1-1*	NA
	6	ST9-5	*MAT1-1-1*	AY004676
	7	ST11-3	*MAT1-1-1*	NA
	8	ST11-37a′	*MAT1-2-1*	NA
	9	ST9-65	*MAT1-1-1*	NA
	10	ST11-63	*MAT1-2-1*	NA
	11	ST9-68	*MAT1-2-1*	NA
	12	ST11-47a	*MAT1-2-1*	NA
	13	ST6-75	*MAT1-2-1*	AY004672
	14	ST9-20	*MAT1-2-1*	AY004673
	15	ST10-10b	*MAT1-2-1*	NA
	16	ST11-10b	*MAT1-2-1*	NA
	17	ST11-67	*MAT1-1-1*	NA
	20	ST11-99	*MAT1-1-1*	AY004677
	21	ST11-94a	*MAT1-1-1*	NA
	22	ST11-27a	*MAT1-1-1*	NA
	23	ST11-86′	*MAT1-2-1*	NA
	24	ST11-17′	*MAT1-2-1*	NA
	25	ST11-76b	*MAT1-2-1*	NA
	26	ST11-1	*MAT1-1-1*	NA
	27	ST6-36	*MAT1-2-1*	NA
	28	ST10-17	*MAT1-2-1*	NA
	29	ST10-26	*MAT1-2-1*	NA
	30	ST9-58	*MAT1-1-1*	Na
	31	ST6-68b	*MAT1-2-1*	AY004674

Identification of mating type genes

Mating types of isolates of *Alternaria brassicae*, *A. brassicicola* and *A. tenuissima* were determined using PCR amplification. Primers were designed from GenBank® sequences from *A. gaisen* (Table 3, Fig. 2) and their sequences followed by their nucleotide position relative to the *A. gaisen* sequences are given below. Primers designed to produce a 938 bp PCR fragment from the *MAT1-1-1* gene had the following sequences:

BPHO4:
5′-TGC CTT TGT TGG ATT TCG GTG TAA G-3′
824-848

BPHO5:
5′-CCG GAG CGG AAA GCA TCG AAG TCG-3′
1761-1738

Table 2. List of species with GenBank® accession numbers for ITS sequences used in this study.

Teleomorph	Anamorph	Isolate[a]	GenBank® accession no. (ITS)
Allewia eureka	*Embellisia eureka*	EGS 36-103	AF392989
A. proteae	*E. proteae*	EGS 39-031	AF392990
Lewia infectoria	*Alternaria infectoria*	–	AF229480
L. photistica	*A. photistica*	–	AF081455
L. scrophulariae	*A. conjuncta*	EGS 37-139	AF392988
L. ethzedia	*A. ethzedia*	EGS 37-143	AF392987
Pleospora herbarum	*Stemphylium botryosum*	–	AF229479
Unknown	*Alternaria alternata*	–	AF229461
Unknown	*A. alternata* GB1	–	U05195
Unknown	*A. alternata* GB2	–	AF071346
Unknown	*A. brassicae* GB1	–	U05253
Unknown	*A. brassicae* GB2	–	AF229463
Unknown	*A. brassicae*	gz01-3-1	AF392983
Unknown	*A. brassicae*	gz01-6-6	AF392984
Unknown	*A. brassicicola*	–	AF229462
Unknown	*A. brassicicola* GB1	–	U05198
Unknown	*A. brassicicola* GB2	–	AF201964
Unknown	*A. brassicicola*	gz01-2-1	AF392985
Unknown	*A. brassicicola*	gz01-8-6	AF392986
Unknown	*A. carotiincultae*	–	AF229465
Unknown	*A. cheiranthi*	–	AF229457
Unknown	*A. crassa*	–	AF229464
Unknown	*A. dauci*	–	AF229468
Unknown	*A. japonica*	–	AF229474
Unknown	*A. macrospora*	–	AF229469
Unknown	*A. petroselini*	–	AF229454
Unknown	*A. porri*	–	AF229470
Unknown	*A. porri* GB1	–	AB026159
Unknown	*A. radicina*	–	AF307015
Unknown	*A. selini*	–	AF229455
Unknown	*A. smyrnii*	–	AF229456
Unknown	*A. solani*	–	AF229475
Unknown	*A. solani* GB1	–	AF314576
Unknown	*A. solani* GB2	–	U80204
Unknown	*A. tenuissima*	ST8-42	AF494276
Unknown	*A. tenuissima*	ST9-20	AF494275
Unknown	*Ulocladium alternariae*	–	AF229485
Unknown	*U. atrum*	–	AF229486
Unknown	*U. botrytis*	–	AF229487
Unknown	*U. chartarum*	–	AF229488

[a] Isolate numbers provided only if the corresponding sequence was determined in this study.

Primers AAM1-2 and AAM1-3 from Arie *et al.* (2000) were used to amplify a 689 bp fragment of the *MAT1-1-1* of *Alternaria brassicicola*. In addition, primer CHO13, the slightly modified complement of Arie *et al.*'s (1997) primer HO13 was used to amplify the 5′ region flanking the *MAT1-1-1* gene.

CHO13:
5′-ATT GCA GAT TGG AAA GGC CAA GT-3′ 176-198

Primers to amplify a 417 bp fragment of the *MAT1-2-1* gene had the following sequences:

BPHMG1:
5′-CAC ACT CTT GTA AAG ATG CC-3′ 1316-1335
BPHMG2:
5′-CTC GCC AGG TTT CCT GGG AG-3′ 1732-1713

For the *MAT1-2-1* gene fragment of the *A. brassicae* and *A. brassicicola* isolates the following primers were used:

MCHMG1:
5′-AAG GCT CCT CGA CCG ATG AAC TG 1445-1467
MCHMG2:
5′-CTG GGR GTR TAC TTG TAG TCR GG 1719-1697

Additional sequences for amplification and sequencing of the *MAT1-2-1* gene fragment included (Fig. 2):

MCH0F:
5′-GGC TAT TTC CTT TGT CTC ATA-3′ 580-600
ATEN1F:
5′-AGC CCT TCT CAC TTG CAC TG-3′ 1144-1163

Table 3. Mating type gene sequence accession numbers.

Species	GenBank® nucleotide accession no.	Mating type gene
Cochliobolus carbonum	AF032368	*MAT1-1-1*
C. ellisii	AF129746	*MAT1-1-1*
C. ellisii	AF129747	*MAT1-2-1*
C. heterostrophus	AF029913	*MAT1-1-1*
C. heterostrophus	X68398	*MAT1-2-1*
C. sativus	AF275373	*MAT1-1-1*
C. sativus	AF275374	*MAT1-2-1*
C. victoriae	AF032369	*MAT1-2-1*
Gibberella moniliformis	AF100925	*MAT1-1-1*
G. moniliformis	AF100926	*MAT1-2-1*
G. zeae	AF318048	*MAT1-1-1*
G. zeae	AF318048	*MAT1-2-1*
Mycosphaerella graminicola	AF440399	*MAT1-1-1*
M. graminicola	AF440398	*MAT1-2-1*
Neurospora crassa	M33876	mt A-1 (*MAT1-1-1*)
N. crassa	M54787	mt a-1 (*MAT1-2-1*)
Pyrenopeziza brassicae	AJ006073	*MAT1-1-1*
P. brassicae	AJ006072	*MAT1-2-1*
A. gaisen	AB009451	*MAT1-1-1*
A. gaisen	AB009452	*MAT1-2-1*

ATEN1R:
5′-CCA GCC GTC AGT GCA AGT GAG AAG-3′
1171-1148

PCR reactions were performed with pre-mixed PCR beads (Amersham Pharmacia 'Ready To Go™'). Thermocycles began with an initial 5 min denaturation at 95 °; followed by 25–30 cycles denaturation for 1 min at 95 °, annealing for 1 min at 55 °, and elongation initially for 45 s at 72 °, extended by 4 additional s per cycle; with a final, 7 min elongation period at 72 ° at the end of the set of cycles.

To establish which mating type gene was present, three separate PCR amplification reactions were performed on genomic DNA from each isolate. Two amplifications were directed toward the mating type genes, one targeting a fragment of *MAT1-1-1* and one targeting a fragment of *MAT1-2-1*. The final amplification targeted a fragment of the glyceraldehyde-3-phosphate dehydrogenase gene (Berbee *et al.* 1999) or ITS region as a positive control for DNA quality. The ITS regions of fungal isolates were amplified and sequenced using primers ITS5 and ITS4 (White *et al.* 1990).

MAT1-1-1 fragments were initially amplified with primers CHO13 and BPHO5, with BPHO4 and BPHO5, or with AAM1-2 and AAM1-3 (Fig. 2). The fragments of the expected size were purified by excising them from agarose minigels re-amplified, and purified for sequencing by ethanol precipitation. *MAT1-2-1* fragments were initially amplified with primers CHO13 and BPHMG2 or with MCHMG1 and MCHMG2. The expected fragments from MCHMG1 and MCHMG2 were purified and internal fragments were sequenced directly. Fragments from CHO13 and BPHMG2 were re-amplified with the primer pairs MCH0F and BPHMG2; ATEN1F and BPHMG2; BPHMG1 and BPHMG2; and CHO13 and ATEN1R and purified for sequencing. Sequences were obtained using the Perkin Elmer Applied Biosystems AmpliTaq DNA polymerase based BigDye™ terminator kit following manufacturer's instructions. Sequences were determined in both directions and were analyzed on an Applied Biosystems Prism 377 DNA sequencer. Sequences of amplified fragments were subjected to BLAST searches. Accession numbers for sequences determined in this study are given in Tables 1 and 2.

Phylogenetic analysis

Using ClustalW 1.74, we aligned sequences (of about 160 amino acids in length) inferred from our *Alternaria MAT1-1-1* fragments (Table 1), along with sequences from 10 other filamentous ascomycetes (Table 3). We then repeated the alignment process for our *MAT1-2-1* sequences (of about 70 amino acids in length). The amino acid sequences of mating type genes were divergent, even within the filamentous ascomycetes. For analysis, we used the most conserved middle regions of the genes, adding gaps as necessary to maximize sequence similarity. For the *MAT1-1-1* sequences, we analyzed 184 aligned positions, corresponding to amino acids 65-240 in *A. gaisen* and for the *MAT1-2-1* sequences, we analyzed 125 aligned positions corresponding to amino acids 132-241 in *A. gaisen* (Fig. 2). For the phylogenies and the bootstrap analysis, the PHYLIP 3.6 (Felsenstein 2001) computer program Protdist calculated the distance matrices using a Jones–Taylor–Thornton substitution model of amino acid substitution. PHYLIP's Fitch produced the phylogenies, using a global search with five replicates and jumbled taxon input order. The bootstrap percentages are the consensus of 500 replicated searches using the global search option in Fitch.

We aligned our ITS sequences manually, beginning with an alignment from TreeBASE ⟨http://herbaria.harvard.edu/treebase/submit.html⟩ (accession number S477) from Pryor & Gilbertson's (2000) study. We chose *Pleospora herbarum* as an outgroup. To minimize alignment problems, we excluded the more divergent sequences from outside of the *Alternaria* and *Pleospora* clades. We used PHYLIP's Dnaml to produce a maximum likelihood tree, to perform a bootstrap analysis, and to estimate a model for substitution. To speed up analysis, we included only one isolate to represent each group of several isolates with identical ITS sequences. Through trial and error, using Dnaml we optimized the sizes of patches of sequence data that would be expected to evolve at the same rate; the coefficient of variation; and the proportion of invariant sites. With these optimized parameters, Dnaml produced a maximum likelihood tree that included all taxa. With the program PAUP*, we repeated the maximum likelihood analysis, again estimating rate parameters. With the program PAUP*, we performed 500 fast (without

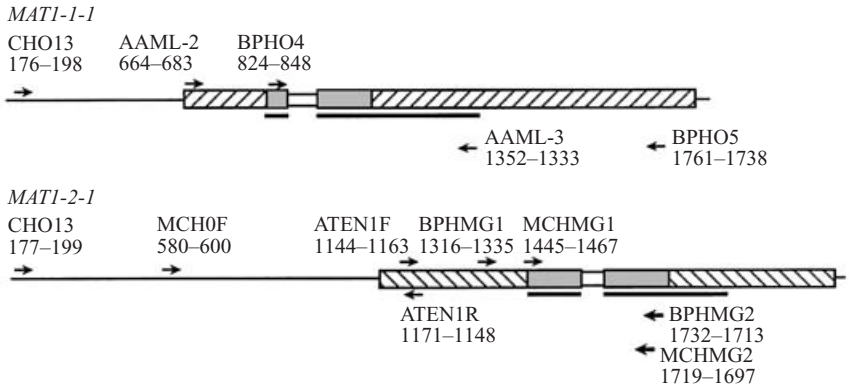

Fig. 2. Schematic diagram of the primers sites and their nucleotide positions, relative to two *Alternaria gaisen* mating type genes, *MAT1-1-1* (GenBank® accession no. AB009451) and *MAT1-2-1* (GenBank® accession no. AB009452). Cross hatched and shaded boxes represent coding regions, and clear boxes represent introns. The shaded box of the *MAT1-1-1* gene represents sequence coding for alpha box of the *MAT1-1-1* region and the shaded box of the *MAT1-2-1* represents sequence coding for the high mobility group box. The underlining indicates the region of each gene used for the amino acid sequence alignment and for the construction of the trees in Fig. 5.

Table 4. Apomorphy list, showing the ITS substitutions that map most parsimoniously to branches leading to *Alternaria brassicae*, *A. brassicicola* and to the *A. tenuissima* and *A. alternata* isolates.

Branch	Site in alignment	Consistency index	Change	Rate-of-change category[a]
To *A. brassicae*				
	27[b]	1	G⇒A	1
	33	0.667	G⇒A	2
	71[b]	0.667	G⇒A	2
	74	0.6	T→C	2
	164	0.667	A→T	2
	184[b]	1	C⇒A	1
	205	0.286	C⇒A	3
	420	0.333	C⇒T	2
	508	0.5	C⇒A	2
	520[b]	1	C⇒T	1
To *A. brassicicola*				
	55[b]	1	T⇒C	2
	56[b]	1	G⇒T	2
	526	0.5	C⇒T	2
To *A. tenuissima* and *A. alternata*				
	40	0.25	T⇒C	2
	107[b]	1	G⇒T	1
	420	0.333	C⇒T	2
	493[b]	1	G⇒A	1
	500[b]	1	T⇒A	2
	501[b]	1	C⇒T	2
	508	0.5	C⇒A	2
	526	0.5	C⇒T	2
	527[b]	0.75	A⇒T	2

[a] Four rate-of-change categories were possible for these variable sites. Sites in Category 1 are expect to change most slowly and sites in Category 4 change most quickly.

[b] The nucleotide at each of these sites was a derived character shared only by the terminal taxa united by the branch under consideration. For example, only the *A. brassicae* isolates had an 'A' at site 27 in the alignment. For site 71, only the *A. brassicae* isolates had an 'A' and other species had a 'G' or a 'C' at the site.

branch swapping) parsimony bootstrap replicates. Also with PAUP*, we performed 500 neighbour-joining bootstrap replicates with distances calculated using a general time-reversible substitution model, assuming 40 % of sites were invariant and that rate variation with gamma-distributed, with a shape parameter of 0.27.

Estimation of ITS substitution probability

To estimate the probability of convergent substitutions on the branch shared by isolates of opposite mating type in the same species, we used the Dnaml maximum likelihood estimates of substitutions per site for each branch. The program Dnaml then provided the rate category, for each site in the alignment, that contributed most to the overall likelihood. We assumed that rates were gamma-distributed across sites, and allowed four different relative rates of substitution for the variable sites, as well as a class of invariant sites.

Using PAUP* we estimated which DNA substitutions would, most parsimoniously, have been gained on branches leading to *Alternaria brassicae*, *A. brassicicola* and the complex of species with identical ITS regions that includes *A. alternata* and *A. tenuissima* (Table 4). Some of the substitutions were at sites that had low consistency indices, indicating conflicting information in the data set and warning of possible error in phylogenetic reconstruction. To be conservative in our probability estimates, we used only sites that had a high consistency index and only substitutions that were unique to the isolates under investigation. We based our probability estimates on these sites (Fig. 3). To calculate the chances of two or more substitutions at the same site, we assumed a Poisson distribution of substitutions as in Burt *et al.* (1996). Where μ is the estimated, category-specific substitution rate per site:

Probability of two or more changes at a site is $(1-e^{-\mu}-\mu e^{-\mu})$.

Probability of two or more changes, given at least one change is $(1-e^{-\mu}-\mu e^{-\mu})/(1-e^{-\mu})$.

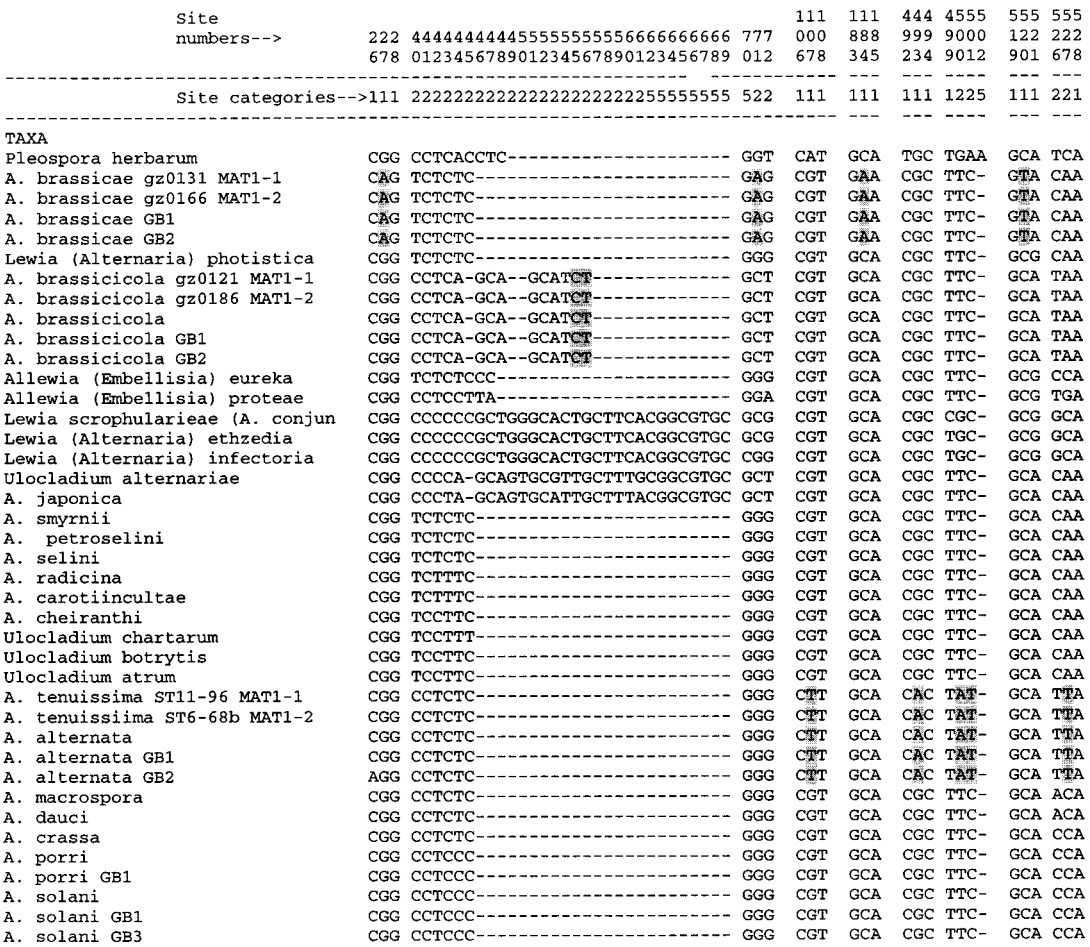

Fig. 3. Portions from the ITS sequence alignment showing, in grey shading, the unique nucleotide substitutions shared only by the *Alternaria brassicae*, the *A. brassicicola* or the *A. tenuissima* plus *A. alternata* isolates. Isolates of different mating types, for all three fungal groups had identical ITS sequences. Site numbers are read vertically and are given in the first three rows (e.g. the first site shown was site 26 in the original alignment). Site categories are the rate-of-change categories that PHYLIP's Dnaml assigned to each site. Sites with the slowest rate of change were in Category 1 and the most rapidly changing sites were in Category 4. Sites in Category 5 were predicted to be invariant.

RESULTS

Mating type gene fragment amplification and analysis

A PCR fragment corresponding to the expected size of one or the other mating type gene was unambiguously amplified from each of the isolates of *Alternaria tenuissima* (Fig. 4, Table 1), *A. brassicae* and *A. brassicicola* (Table 1). Isolates that produced a *MAT1-1-1* gene fragment did not produce a PCR product with the *MAT1-2-1* primers BPHMG1 and BPHMG2. Isolates that produced a *MAT1-2-1* PCR fragment often gave a weak amplification product, though not of the expected size for the *MAT1-1-1* gene product (Fig. 2), with the *MAT1-1-1* primers BPHO4 and BPHO5 (Fig. 4).

For the *A. brassicae* and *A. brassicicola* isolates, all isolates from the same leaf spot had the same mating type. The 18 isolates of *A. brassicicola* from the same leaf spot that produced isolate gz01-2-1 all amplified the *MAT1-1-1* gene fragment and the eight isolates from the same leaf spot that produced isolate gz01-8-6 all amplified the *MAT1-2-1* gene fragment (Table 1). All of the nine isolates of *A. brassicae* that included isolate gz01-3-1 amplified the *MAT1-1-1* gene fragment, while all of the eight isolates from the leaf spot that produced gz01-6-6 amplified the *MAT1-2-1* gene fragment (Table 1). Out of 28 isolates from the *A. tenuissima* group, genomic DNA from 13 isolates positively amplified for the *MAT1-1-1* gene and 15 isolates (54%) amplified positively for *MAT-1-2-1* (Fig. 4, Table 1). All negative controls lacking template appeared clean and the *GPD* or ITS positive control amplified positively for all reactions (not illustrated).

The phylogenetic analysis showed the mating type gene fragments determined in this study (from *A. brassicae*, *A. brassicicola* and the *A. tenuissima* group) cluster with known mating type genes with high bootstrap support (Fig. 5). The *Alternaria* and *Cochliobolus MAT1-1-1* genes clustered together with 100% bootstrap support. The *MAT1-1-1* amino acid sequences from the three *Alternaria* species clustered together with a previously determined sequence from *A. gaisen* with 100% bootstrap support (Fig. 5A). The *Alternaria*

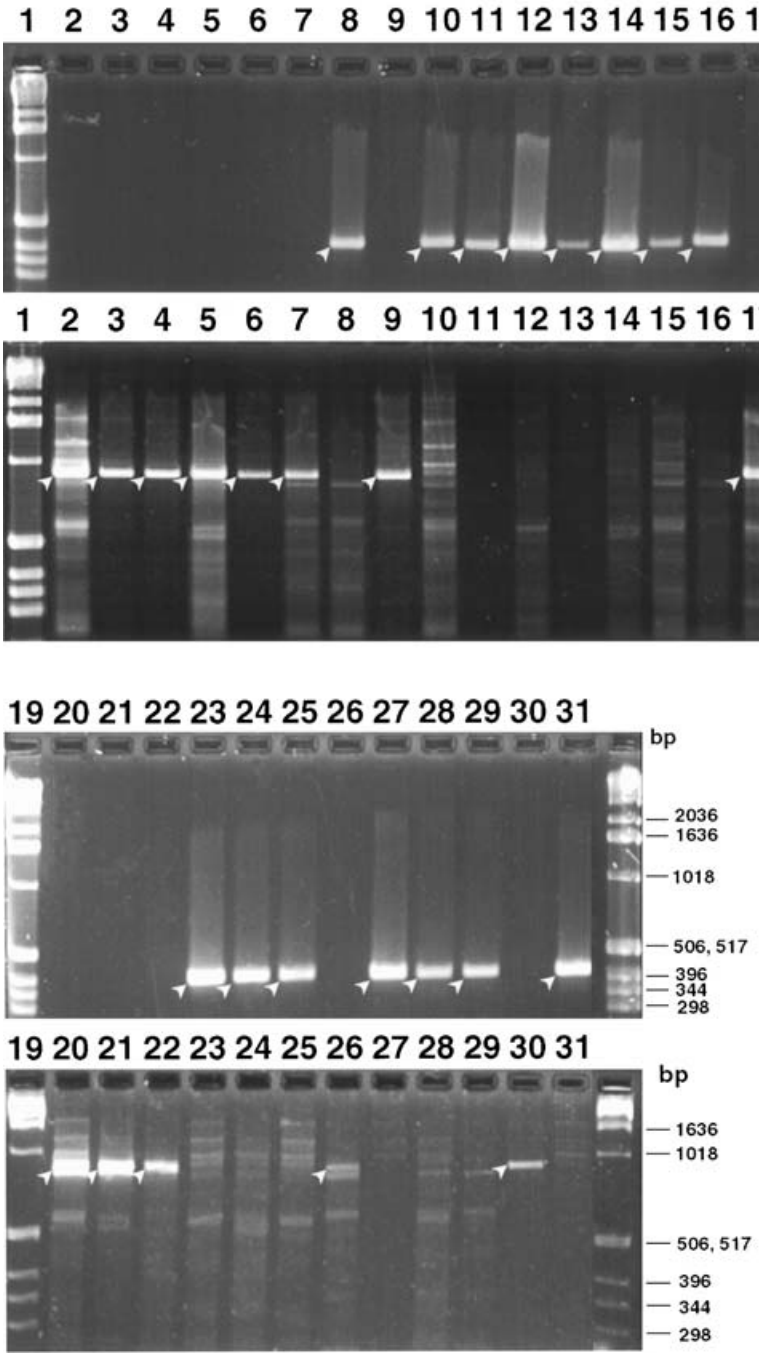

Fig. 4. Amplification of mating type gene fragments from 28 *Alternaria tenuissima* group isolates. The top gel and the third gel show attempted amplifications of a *MAT1-2-1* gene fragment (expected product size, 417 bp) from each isolate. The second and bottom gel show attempted amplification of a *MAT1-1-1* gene fragment (expected product size; 938 bp) from each isolate. Lanes 1, 18, 19, 32: 1 kb ladder (Gibco BRL) molecular weight marker. Lanes 2–17 and 20–31: amplification products from isolates of the *Alternaria tenuissima* group. Lanes with the same number contain PCR product from the same isolate. Lane number corresponds to the isolate number in Table 1. Each isolate produced the expected amplicon (indicated by an arrowhead) of only one of the mating type genes. As an example, in lane 2 of the top gel, *A. tenuissima* isolate 2 gave no *MAT1-2-1* amplification product, while the second gel shows that isolate 2 did give the 938 bp amplification product expected from the *MAT1-1-1* gene.

and *Cochliobolus MAT1-2-1* genes also clustered together with 100% bootstrap support, and the *Alternaria* sequences clustered together with 77% bootstrap support (Fig. 5B).

The DNA sequences and inferred amino acid sequences of the amplified *MAT* gene fragments were highly similar to *MAT* genes from *A. gaisen* from GenBank. The *A. brassicae* and *A. brassicicola MAT1-1-1* DNA sequences were 87–88% identical to the *A. gaisen* sequence and the amino acid sequences were 84–88% identical to the *A. gaisen* sequence. The sequences from *MAT1-1-1* gene fragments from three

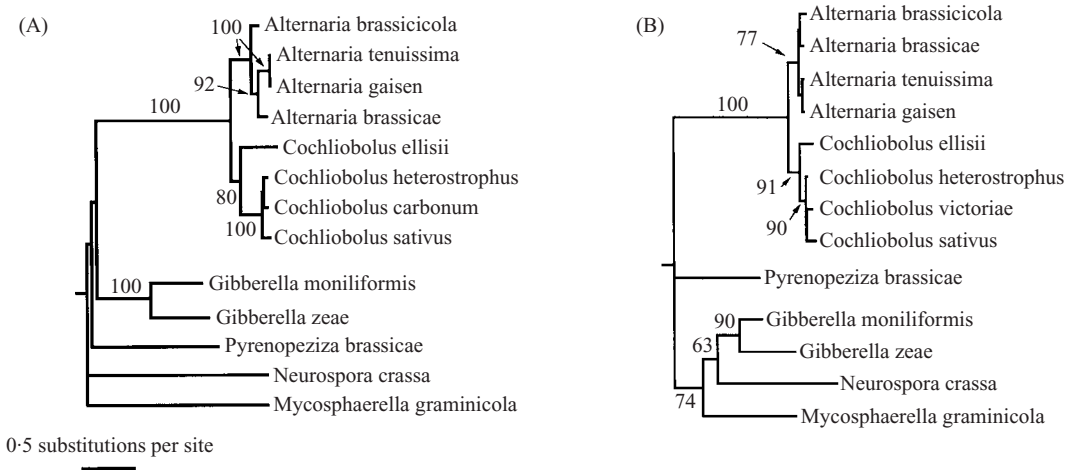

Fig. 5. Phylogenetic relationships of the *MAT1-1-1* (A) and the *MAT1-2-1* (B) homologues based on alignment of conserved amino acid sequences. *Cochliobolus* and *Alternaria* are closely related genera in the *Pleosporaceae*, and function has been demonstrated for the mating type genes in the *Cochliobolus* species and in *Alternaria alternata* (syn. *A. gaisen*). Numbers are bootstrap percentages, from 500 Fitch bootstrap replicates.

A. tenuissima group isolates (Table 1) were identical. The *A. tenuissima* group isolates differed from the GenBank® *MAT1-1-1* sequence from *A. gaisen* at only one position. Similarly, the *MAT1-2-1* sequences of three *A. tenuissima* group isolates (Table 1) were identical across their 581 bp alignment. The three *A. tenuissima* group *MAT1-2-1* sequences differed from the *A. gaisen MAT1-2-1* sequence at eight positions. The *MAT1-1-1* and the *MAT1-2-1* DNA and inferred amino acid sequences from the *A. tenuissima* group isolates were all more than 98% identical to their homologues from *A. gaisen*.

ITS sequence analysis

The ITS sequences from isolates of the opposite mating type but the same species were identical (Fig. 6). Isolates of the same species clustered together in the maximum likelihood tree and with strong neighbour-joining and fast parsimony bootstrap support. None of the species without known sexual states clustered strongly with any of the sexual species in the ITS phylogeny. The six species of *Alternaria* and *Embellisia* with known sexual states (in *Lewia* and *Allewia*, respectively) appear at the base of the tree, but without bootstrap support (Fig. 6).

Model for ITS sequence change

To model DNA substitution rate variation in the ITS region we initially inferred a Dnaml tree assuming that substitution rates were uniform. We then optimized the substitution model, maximizing likelihood of the data given the tree by successive approximation. The likelihood of the tree was highest when we assumed that substitution rates for adjacent sites were correlated and that the mean block length of sites with the same substitution rate was three. Likelihood was also highest when we assumed that 40% of sites were invariant, or in other words, unable to change during the period of evolution represented by the phylogeny. Assuming that rate variation followed a gamma distribution, likelihood was highest when the coefficient of variation was set to two. Allowing for four different rate categories among the 60% of sites that were free to vary, Dnaml estimated that the proportion of sites in the slowest rate category was 0.51 and that these sites evolved at 0.27 times the average rate. The proportion of sites in Category 2 was 8.3×10^{-2} and these sites evolved at 4.55 times the average rate. The proportion of sites in Category 3 was 5.6×10^{-3} and these sites evolved at 14.47 times the average rate. The proportion of sites in Category 4 was vanishingly small (5.9×10^{-5}) and the sites evolved at 32.71 times the average rate. Using this substitution model allowing for rate variation, we again searched for a most likely tree. We verified that the maximum likelihood tree including all isolates and the tree excluding repeated ITS sequences were topologically the same and had the same branch lengths. Dnaml matched each of the variable sites in the alignment with the rate category that contributed most to the likelihood (Fig. 3). Variation did not occur at all of the 60% of sites that were potentially variable. Substitutions were only observed at 117 of the estimated 350 variable sites. As expected, the invariant sites were concentrated in the genes. Dnaml classified all 11 sites in the 18S rRNA gene, 95 out of 158 sites in the 5.8S gene, and 24 of the 34 sites in the 28S gene as invariant. As expected, sites with a high consistency index in parsimony were predicted to have relatively low rates of change by Dnaml (Table 4, Fig. 3).

Estimates of probabilities of origin by convergence for the observed ITS substitutions

The next question was whether the shared ITS substitutions could have originated convergently in clonal isolates of opposite mating type (Fig. 1). We used the

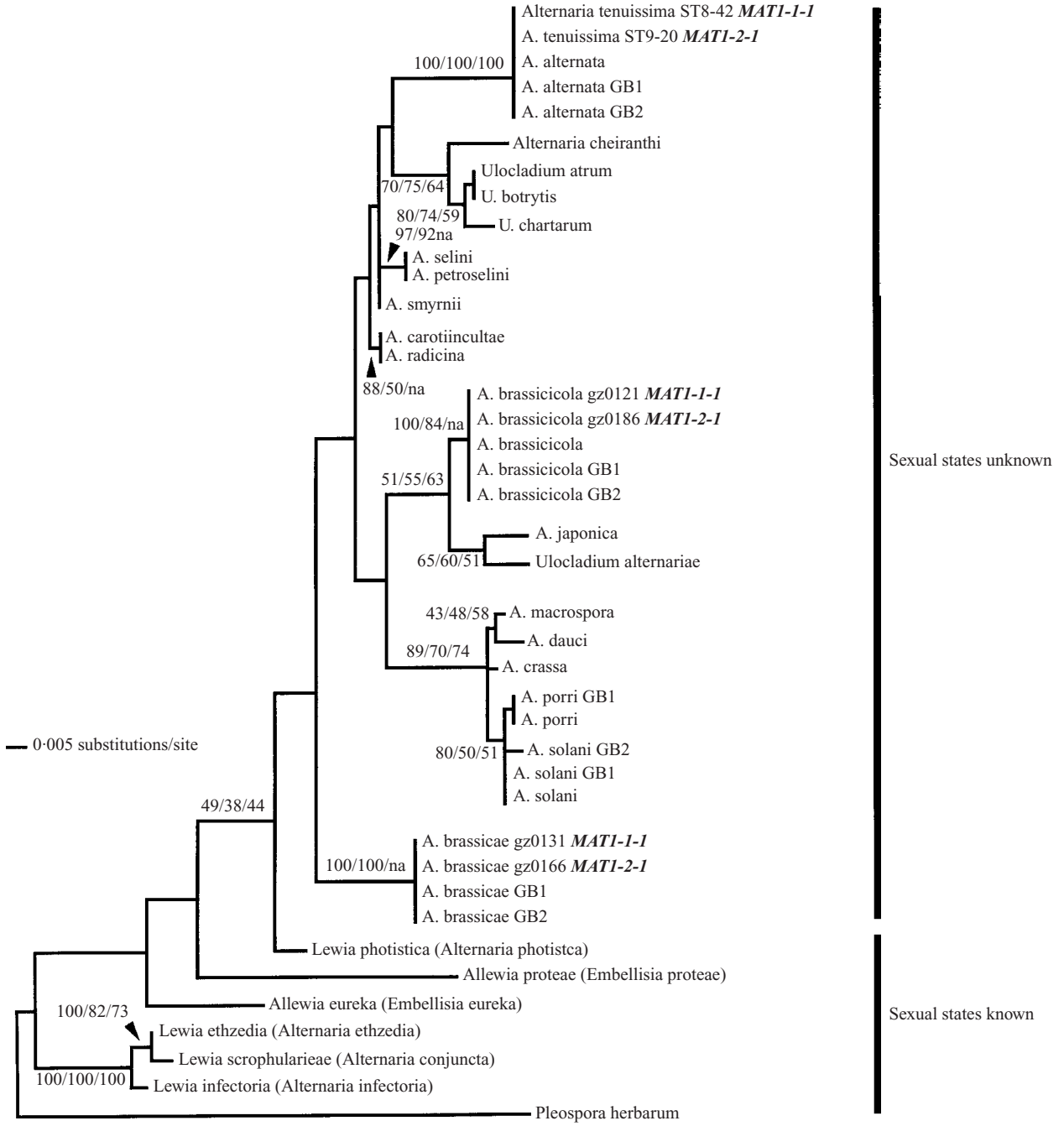

Fig. 6. This maximum likelihood phylogram from ribosomal ITS sequences shows that isolates having the opposite mating type genes *MAT1-1-1* and *MAT1-2-1* were found in three putative asexual species. Branch lengths are proportional to estimated substitutions per site, so the substitutions that accumulated since the most recent speciation event are represented by the length of the horizontal branch leading to the species. Individuals of opposite mating type have identical ITS sequences. Numbers are bootstrap percentages from, respectively, neighbour joining, fast parsimony and maximum likelihood. The 'NA' indicates that maximum likelihood bootstrap support was not calculated for the *Alternaria brassicicola* and *Alternaria brassicae* clusters because only one isolate from each cluster was included in the analysis.

maximum likelihood estimates of branch length and substitution rates for the probability estimates, to allow for the possibility that some of the substitutions shared by individuals of opposite mating type may occur at DNA sites with an unusually high rate of substitution. The maximum likelihood branch length, expressed as average number of substitutions per site over the 584 sites in the alignment, was 0.0206 for the branch uniting the *Alternaria brassicae* isolates; 0.0036 for the branch uniting the *A. brassicicola* isolates; and 0.0209 for the branch uniting the *A. tenuissima* and *A. alternata* isolates. To assess the probability of convergent change (Fig. 3) we took into account that only 60 % of the sites in the alignment were variable. The average substitution rate for the sites that were free to vary equals the average substitution rate across all sites, divided by

Table 5. Calculation of probability of convergent origin for the unique, shared substitutions that map to branches leading to the *Alternaria brassicae*, *A. brassicicola* and to the *A. tenuissima* and *A. alternata* isolates.

Branch in phylogeny and its estimated average number of substitutions per site[a]	Substitution rate category	Relative rate of substitution for each rate category	Proportion of sites in each rate category[b]	Expected substitution number per site (μ) for branch, for each relative rate category[c]	Expected number of substitutions in each rate category for the branch[d]	Probability of two or more changes at same site for sites of each rate category[e]	Probability of two or more changes at same site, given one change at the site[f]	Observed number of synapomorphic substitutions in the rate category[g]	Chances of convergent origin of all synapomorphic substitutions occurring in the rate category
To *Alternaria brassicae* (3.44×10^{-2})	1	0.27	5.1×10^{-1}	9.40×10^{-3}	2.79	4.35×10^{-5}	4.70×10^{-3}	3	1.02×10^{-7}
	2	4.55	8.3×10^{-2}	1.57×10^{-1}	7.58	1.10×10^{-2}	7.62×10^{-2}	1	7.62×10^{-2}
	3	14.47	5.6×10^{-3}	4.98×10^{-1}	1.63	8.95×10^{-2}	2.28×10^{-1}	0	–
	4	32.71	5.90×10^{-5}	1.12	3.88×10^{-2}	3.10×10^{-1}	4.59×10^{-1}	0	–
To *Alternaria brassicicola* isolates (6.00×10^{-3})	1	0.27	5.1×10^{-1}	1.60×10^{-3}	4.86×10^{-1}	1.33×10^{-6}	8.00×10^{-4}	0	–
	2	4.55	8.3×10^{-2}	2.72×10^{-2}	1.32	3.66×10^{-4}	1.36×10^{-2}	2	1.85×10^{-4}
	3	14.47	5.6×10^{-3}	8.67×10^{-2}	2.83×10^{-1}	3.56×10^{-3}	4.28×10^{-2}	0	–
	4	32.71	5.9×10^{-5}	1.96×10^{-1}	6.70×10^{-3}	1.69×10^{-2}	9.49×10^{-2}	0	–
To *Alternaria alternata* and *A. tenuissima* isolates (3.49×10^{-2})	1	0.27	5.1×10^{-1}	9.50×10^{-3}	2.83	4.48×10^{-5}	4.70×10^{-3}	2	2.25×10^{-5}
	2	4.55	8.3×10^{-2}	1.59×10^{-1}	7.70	1.13×10^{-2}	7.73×10^{-2}	3	4.62×10^{-4}
	3	14.47	5.6×10^{-3}	5.05×10^{-1}	1.65	9.17×10^{-2}	2.31×10^{-1}	0	–
	4	32.71	5.9×10^{-5}	1.14	3.93×10^{-2}	3.16×10^{-1}	4.65×10^{-1}	0	–

[a] For 60% of sites that were variable.

[b] The remaining 40% of sites were invariant.

[c] The expected substitution number per site, per branch, for each relative rate category was calculated as the relative rate times average substitutions per variable site.

[d] The expected number of substitutions for the branch, for sites in each rate category was the estimated average number of substitutions per site times 584 sites times the proportion of sites in the rate category. The sum of expected substitutions in all rate categories is the number of substitutions expected during the evolution.

[e] Assuming a Poisson distribution, $P = (1 - e^{-\mu} - \mu e^{-\mu})$, where μ is the category-specific average number of substitutions per variable site.

[f] $P = (1 - e^{-\mu} - \mu e^{-\mu})/(1 - e^{-\mu})$.

[g] Substitutions for sites in specified rate categories, shared only by the isolates united by the branch.

the proportion of variable sites. Taking the branch to *Alternaria brassicae* isolates as an example, the average substitution rate for the sites that were free to vary was 0.0206/0.6 or 3.44×10^{-2} (Table 5). For each branch, we could then estimate the expected number of substitutions per site, for the variable sites in each different rate category. For each rate category, the expected number of substitutions per site was the overall expected substitution per site, for the 60% of sites that were free to vary, times the relative rate for the particular rate category of the site. For the *Alternaria brassicae* example, the relative rate of change, for the sites in Category 1, was 0.272. The average number of substitutions per site for sites that were free to vary was 3.44×10^{-2}. Multiplying 0.272 by 3.44×10^{-2} gives 9.4×10^{-3} as the expected number of substitutions per Category 1 site for the branch (Table 5). The average substitution rates per site, for all rate categories, for all three *Alternaria* species are given in Table 5.

As it should be, the expected total number of substitutions per branch is the same if calculated based on the average substitutions per site, or if it is calculated by summing all the expected numbers of substitutions at sites in each rate category (Table 5).

For example, multiplying 0.0206, the average number of substitutions per site for the branch leading to *Alternaria brassicae* by the number of aligned sites (584) gives the prediction that 12 substitutions would have occurred during the evolution of this branch. The same total results from adding the 2.7918 expected Category 1 substitutions, plus 7.5837 Category 2 substitutions, plus 1.6278 Category 3 substitutions, plus 0.0388 Category 4 substitutions for the branch (Table 5). The maximum likelihood total is higher than the ten substitutions that map to same the branch using parsimony (Table 4) because the likelihood estimate includes an estimate of the number of convergent changes that cannot be detected by parsimony. Two substitutions would be predicted to have occurred on the branch leading to the *A. brassicicola* isolates, and 12.2 on the branch common to the *A. tenuissima* and *A. alternata* isolates (Table 5).

Given one substitution at a site, the probability that a second substitution would have occurred at the same site would be the same as the probability of a single substitution at any site. However, if substitutions are Poisson distributed, there may or may not have been a first substitution to match with a second one. As a result of the Poisson distribution, the probability of two or more substitutions at the same site was about half the probability of one substitution at any site (Table 5).The chances of having two or more changes at the same site, given an initial substitution, would be 4.7×10^{-3} for sites in the slowest substitution rate group, Category 1, and 7.62×10^{-2} for sites in Category 2, for the branch leading to *A. brassicae*. The isolates of opposite mating type in the species share substitutions at three Category 1 sites and one Category 2 site (Fig. 3). The probability of even one such shared substitution occurring convergently is small; the probability of all these substitutions occurring by convergence is negligible (Table 5).

Calculation of convergent substitution probabilities on the branch leading to the *A. tenuissima* and *A. alternata* clades leads to similarly low probabilities for convergent substitutions.

Probabilities of convergent substitution for *A. brassicicola* isolates of opposite mating type are based on a region in the alignment with insertions and deletions (Fig. 3). By adding one more gap to the *A. brassicicola* sequences, these sequences could be realigned so that they differ from the *Lewia* sequences by only the gap. However, no matter how the *A. brassicicola* sequences are aligned, these isolates share at least one insertion, deletion, or substitution, in the region between sites 40 and 69, that distinguishes them from all other species.

DISCUSSION

In fungal species without observed sexual reproduction, the usual way to infer sexual history is to evaluate whether population genetic structure is characteristic of a recombining or non-recombining history. Because the mating type genes themselves cannot have recombined, any characters associated with opposite mating types in a population must have either originated convergently, or been exchanged by recombination. Each haploid isolate of *Alternaria brassicae*, *A. brassicicola* and *A. tenuissima* had just one of the two possible mating types, indicating that the mating type switching that occurs in the yeast *Saccharomyces cerevisiae* is not possible here. Finding shared ITS substitutions in individuals of opposite mating type of three presumed asexual species of *Alternaria* is the first positive evidence for a history of recombination during the evolutionary history of these species. How recently could sexual reproduction have been lost, assuming that it was lost after the last ITS substitution shared between opposite mating types in a species? Where the rate of ITS substitution could be estimated from dated divergences, ITS substitution rates appear to be within an order of magnitude of the substitution rates for synonymous substitutions at protein coding genes, that is, about 1×10^{-9} substitutions per site per year (Li & Graur 1991, Sang *et al.* 1995, Chiang & Schaal 2000, Hibbett 2001). Assuming that the rate of ITS substitutions was 1×10^{-9} substitution per site per year, the *Alternaria* species, with a total of about 335 nucleotides in the ITS1 and ITS2 regions, would be expected to acquire one substitution every 3 Myr. The 12 substitutions predicted for the branch to the *A. brassicae* or to the *A. tenuissima* isolates represent perhaps 36 Myr of recombining history; the two substitutions on the branch leading to the *A. brassicicola* isolates represent 6 Myr of recombining history. The limit of our data is that we can only speak about conditions in an ancestral population 3 Myr ago and cannot prove that sex is occurring in any populations now.

In our phylogenetic analysis, we included all *Alternaria* and *Embellisia* species with known sexual states. In the ITS phylogeny, the species with known sexual states appear basal, although without bootstrap support (Fig. 6), as would have been consistent with a radiation of asexual taxa in *Alternaria*. Finding that all ITS substitutions are shared across mating types in three asexual species contradicts the interpretation that an asexual radiation took place. The shared substitutions indicate instead that their evolutionary history, shared through the filamentous *Ascomycota*, was sexual (in some form) and any asexual evolutionary component was too recent to be recorded in ITS region substitutions.

The ITS regions are usually homogeneous within species, so comparison of ITS variation with mating type genes is likely to indicate that a species had a sexual ancestor but not whether sexual reproduction has occurred within a species. Mating type gene comparisons might also be useful in showing that recombination has occurred within species, if future studies adding more variable genetic loci to an analysis show that within-species polymorphisms are shared across mating types.

Species delimitation among *Alternaria alternata* and its close relatives remains controversial (Kusaba & Tsuge 1995, Roberts, Reymond & Andersen 2000, Andersen *et al*. 2001) and ITS regions are probably not variable enough to reveal the boundaries of a species. All of the ITS sequences in our alignment from *A. alternata* and from the *Alternaria tenuissima* group were identical. However, Roberts *et al*. (2000) analyzed RAPD banding patterns to show that *A. alternata* isolate EGS 34-016 (source of ITS sequence AF071346 used in this study, Table 2) was close to other isolates of *A. alternata* but was genetically distant from *A. tenuissima* group isolates ST8-42 and ST9-20 (the sources of ITS sequences AF494276 and AF494275 used in this study, Table 2). If the ITS regions are not variable enough to show that *A. tenuissima* is a distinct species, they cannot be variable enough to show whether the *A. tenuissima* group isolates had a sexual history, independent of a common ancestor with *A. alternata*.

Our study includes only 20 of the 50 or more species of *Alternaria*. Increasing the number of *Alternaria* species sampled, without also increasing the amount of sequence data per taxon, is likely to result in shorter branch lengths because some of the substitutions formerly considered to be unique to one species would turn out to be shared between two or more species. The shorter the branch in the tree, the lower the estimate for the probability of convergent substitution (because less time elapsed, since divergence from a common ancestral species, for substitutions to accumulate) but the greater the difficulty in finding substitutions unique to a particular species.

The branch uniting the *A. brassicicola* isolates is a good example of a short branch. Both mitochondrial SSU and ITS sequences show that *A. brassicicola* is closely related to two other species, *A. japonica* and *Ulocladium alternariae* (Pryor & Gilbertson 2000). The ITS sites that unite the *Alternaria brassicicola* isolates are in a region of the alignment with a history of insertions and deletions (indels). More data, perhaps from protein coding genes, where indels are less common, would be needed for a good estimate of the probability of independent origin of shared change for this species. However, difficult though it is to model their probability, indels are less common than nucleotide substitutions. No matter how the *A. brassicicola* sequences are realigned in the region between sites 40 and 69, they share at least one indel that distinguishes them from all other species and supports a recombining history for individuals of opposite mating type, since their divergence from the *A. japonica* and *U. alternariae* clade.

In conclusion, we have amplified and sequenced opposite mating type gene fragments from the supposed asexual isolates of *A. brassicae*, *A. brassicicola* and of the *A. tenuissima* group. In each species, isolates of opposite mating type had identical ITS regions. The shared ITS variation among isolates of opposite mating type is evidence for a previously concealed evolutionary history of active recombination. Searching for recombination across opposite mating types will be broadly useful for finding evidence of sexual reproduction during evolution in the *Ascomycota*.

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REFERENCES

Andersen, B., Krøger, E. & Roberts, R. G. (2001) Chemical and morphological segregation of *Alternaria alternata*, *A. gaisen* and *A. longipes*. *Mycological Research* **105**: 291–299.

Arie, T., Christiansen, S. K., Yoder, O. C. & Turgeon, G. G. (1997) Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved *MAT* HMG box. *Fungal Genetics and Biology* **21**: 118–130.

Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y. & Yamaguchi, I. (2000) Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata*. *Molecular Plant–Microbe Interactions* **13**: 1330–1339.

Berbee, M. L., Pirseyedi, M. & Hubbard, S. (1999) *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia* **91**: 964–977.

Burt, A., Carter, D. A., Koenig, G. L., White, T. J. & Taylor, J. W. (1996) Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. *Proceedings of the National Academy of Sciences, USA* **93**: 770–773.

Caten, C. E. (1981) Parasexual processes in fungi. In *The Fungal Nucleus* (K. Gull & S. G. Oliver, eds): 191–214. Cambridge University Press, Cambridge, UK.

Centraalbureau voor Schimmelcultures (1990) *List of Cultures, Fungi and Yeasts*. 32 edn. CIP-Gegevens Konninklijke Bibliotheek, Den Haag.

Chelkowski, J. & Visconti, A. (eds) (1992) *Ecological and Evolutionary Roles of Toxins from Alternaria Species Pathogenic to Plants. Alternaria: biology, plant diseases and metabolites*. Elsevier Science Publishers, Amsterdam.

Chiang, T.-Y. & Schaal, B. A. (2000) The internal transcribed spacer 2 region of the nuclear ribosomal DNA and the phylogeny of the moss family *Hylocomiaceae*. *Plant Systematics and Evolution* **224**: 127–137.

Clutterbuck, A. J. (1996) Parasexual recombination in fungi. *Journal of Genetics* **75**: 281–286.

Dugan, F. M. & Roberts, R. G. (1994) Etiology of preharvest colonization of bing cherry fruit by fungi. *Phytopathology* **84**: 1031–1036.

Felsenstein, J. (2001) *PHYLIP: phylogeny inference package*. Version 3.6a2. Distributed by the author, Department of Genetics, University of Washington, Seattle.

Geiser, D. M., Pitt, J. I. & Taylor, J. W. (1998) Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proceedings of the National Academy of Sciences, USA* **95**: 388–393.

Graeser, Y., Volovsek, M., Arrington, J., Schoenian, G., Presber, W., Mitchell, T. G. & Vilgalys, R. (1996) Molecular markers reveal that population structure of the human pathogen *Candida albicans* exhibits both clonality and recombination. *Proceedings of the National Academy of Sciences, USA* **93**: 12473–12477.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C. & Pegler, D. N. (1995) *Ainsworth & Bisby's Dictionary of the Fungi*. 8th edn. CAB International, Wallingford.

Hibbett, D. S. (2001) Shiitake mushrooms and molecular clocks: historical biogeography of *Lentinula*. *Journal of Biogeography* **28**: 231–241.

Hull, C. M. & Johnson, A. D. (1999) Identification of a mating type-like locus in the asexual pathogenic yeast *Candida albicans*. *Science* **285**: 1271–1275.

Kusaba, M. & Tsuge, T. (1995) Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. *Current Genetics* **28**: 491–498.

Lee, S. B. & Taylor, J. W. (1990) Isolation of DNA from fungal mycelia and single spores. In *PCR protocols, a guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 282–287. Academic Press, San Diego, California.

Li, W.-H. & Graur, D. (1991) *Fundamentals of Molecular Evolution*. Sinauer Associates, Sunderland MA.

Metzenberg, R. L. & Randall, T. A. (1995) Mating type in *Neurospora* and some closely related ascomycetes: some current problems. *Canadian Journal of Botany* **73** (Suppl. 1): S251–S257.

Pryor, B. M. & Gilbertson, R. L. (2000) Molecular phylogenetic relationships amongst *Alternaria* species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. *Mycological Research* **104**: 1312–1321.

Roberts, R. G., Reymond, S. T. & Andersen, B. (2000) RAPD fragment pattern analysis and morphological segregation of small-spored *Alternaria* species and species groups. *Mycological Research* **104**: 151–160.

Sang, T., Crawford, D. J., Stuessy, T. F. & O. Silva, M. (1995) ITS sequences and the phylogeny of the genus *Robinsonia* (*Asteraceae*). *Systematic Botany* **20**: 55–64.

Sharon, A., Yamaguchi, K., Christiansen, S., Horwitz, B. A., Yoder, O. C. & Turgeon, B. G. (1996) An asexual fungus has the potential for sexual development. *Molecular and General Genetics* **251**: 60–68.

Shiu, P. K. T. & Glass, N. L. (2000) Cell and nuclear recognition mechanisms mediated by mating type in filamentous ascomycetes. *Current Opinion in Microbiology* **3**: 183–188.

Simmons, E. G. (1986) *Alternaria* themes and variations (22–26). *Mycotaxon* **25**: 287–308.

Simmons, E. G. (1990) *Embellisia* and related teleomorphs. *Mycotaxon* **38**: 251–265.

Taylor, J. W., Jacobson, D. J. & Fisher, M. C. (1999) The evolution of asexual fungi: reproduction, speciation and classification. *Annual Review of Phytopathology* **37**: 197–246.

Turgeon, B. G. (1998) Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology* **36**: 115–137.

Turgeon, B. G. & Berbee, M. L. (1998) Evolution of pathogenic and reproductive strategies in *Cochliobolus* and related genera. In *Molecular Genetics of Host-Specific Toxins in Plant Disease* (K. Kohmoto & O. C. Yoder, eds): 153–163. Kluwer Academic Publishers, Dordrecht.

Turgeon, B. G., Bohlmann, H., Ciuffetti, L. M., Christiansen, S. K., Yang, G., Schäfer, W. & Yoder, O. C. (1993) Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus*. *Molecular and General Genetics* **238**: 270–284.

Turgeon, B. G. & Yoder, O. C. (2000) Proposed nomenclature for mating type genes of filamentous ascomycetes. *Fungal Genetics and Biology* **31**: 1–5.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. Academic Press, San Diego.

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